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Reducing substances in milk with particular reference to the methylene blue reduction test. 1935.

REDUCING SUBSTANCES IN MILK WITH PARTICULAR REFERENCE TO THE METHYLENE BLUE REDUCTION TEST.

by

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A thesis submitted from the Department of Biochemistry
in partial fulfilment of the requirements for
the degree of Master of Science at the
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CONTENTS

The author is deeply indebted to Professor G. Hunter and to Professor H. R. Thornton for their helpful criticism of this work during its progress.

The cooperation of Professor E. Boomer is gratefully acknowledged.

INTRODUCTION

The methylene blue reduction test is used extensively as a method of milk control and is one of the three standard methods recognized by the American Public Health Association. Comparison with the other tests commonly used, with particular reference to accuracy, justify this extensive use. The intelligent interpretation of results from any test requires knowledge of its underlying principles. There are factors which affect the reduction of methylene blue by milk whose importance has been either under estimated or overlooked. While bacteria must play a predominating role, indeed the utility of the test depends on this, their presence is not necessary for the reduction of the dye by milk. Indeed it is probable that even in the presence of bacteria the mechanism of reduction is caused by a number of contributing factors. These factors have specific optimum conditions. Factors which cause reduction in one particular instance, may play but little part in the reduction process in another. A more thorough knowledge of these factors is necessary and is the object of this investigation.

REVIEW

The many theories advanced to explain the reduction of methylene blue in milk go back to Helmholtz (1844) who observed the reduction of litmus by bacteria. Since that time the reduction of dyes by bacteria has been much studied. Fred (1912) compiled an extensive bibliography of the work done in this field: while Clark, Cohen and Gibbs (1925) carried out extensive investigations on methylene blue as an indicator of oxidation-reduction phenonena. From Neisser and Wechsberg's (1900) suggestion that methylene blue might be used to determine the bacterial content of milk, the Standard Methylene blue test has been elaborated. Shardinger (1902) demonstrated rapid reduction of a methylene blue-formaldehyde mixture in unheated milk; heated milk failed to show such rapid reduction.

The earlier conception was that the reduction of methylene blue in milk is dependent on an enzyme supposedly elaborated by the growing bacteria and termed "reductase" - hence the test was called "The Reductase Test" - a term which implies that reduction is accomplished enzymatically, for which there is insufficient evidence. This theory is no longer tenable since reduction has been demonstrated in milk where bacterial action is not a factor. Burri and Kürsteiner (1912) showed that reduction of methylene blue takes place in fresh milk in which bacterial action has been inhibited by antiseptics and more recently Barthel (1925) demonstrated rapid reduction of methylene blue in de-aerated fresh milk of low bacterial content, in de-aerated sterile milk and in synthetic milk. Barthel (1917) advanced the theory that the reduction of methylene

blue in milk takes place in two stages:-

(1) The removal of the dissolved oxygen by the growing bacteria.

(2) The reduction of the dye by constituents of the milk.

He later postulated the citrate in the milk to be the hydrogen acceptor and that the action was catalysed by the milk salts. Viale (1927) claims he demonstrated the presence of a sulphydryl group in milk and assumes it to be responsible for reduction. Clark, Cohen and Gibbs (1925) demonstrated the use of methylene blue as an oxidation-reduction indicator and followed the reduction of methylene blue in milk potentiometrically. This opened up a new avenue of approach and Thornton and Hastings (1929,1930) made a series of determinations on oxidation-reduction potentials in milk. They found that methylene blue in milk reduced over a slightly higher potential range than that reported by Clark et al and attributed the result to salt effect or the influence of other oxidation-reduction systems. They showed that the typical potential range of milk is not appreciably affected by the addition of methylene blue in the dilution used in the standard test, thus the dye may be considered as acting as an oxidation-reduction indicator. They confirmed Barthel's theory that the reduction of the dye takes place in two stages. Schwarz (1929) suggested that sulphydryl systems produced by degradation of the protein or as end products of bacterial metabolism might be causing reduction. Whitehead (1930) observed the catalytic effect of sunlight on the reduction of methylene blue in milk and suggested that this was due to an oxidation-reduction reaction in which unsaturated fats are oxidised, the methylene blue acting as a hydrogen acceptor. Martini (1931) claimed to have isolated gluthathione

from milk and believes it becomes oxidised in the presence of sunlight and that methylene blue is reduced in the process.

From all this confusion one fact becomes immediately self-evident, that although reduction of methylene blue takes place in sterile milk, the presence of bacteria affects the reduction and when present in very large numbers must become of major importance. The reducing power of bacteria is too well established to need any further comment and is excellently reviewed by Hewitt (1933). A number of organisms which commonly occur in milk will produce potentials more negative than the range of methylene blue which indeed is a very narrow one . While there is a high correlation between numbers of bacteria and reduction times instances occur where no correlation exists; this fact will be recognized by those with practical experience of this test. Skar (1913, 1931) has attempted to explain the discrepancy and attributes it to the reducing power of the leucocytes and epithelial debris commonly present in milk. This discrepancy in correlation between either the number of bacteria or the number of leucocytes and the reduction times in milks, has been widely observed and has been confirmed by The Department of Dairying, University of Alberta, where it has been the subject of an investigation by N. J. Strynadka who has done extensive microscopic work along this line. Through his courtesy the figures in the first table are presented:

Table 1. The methylene blue reduction times and the bacterial and leucocyte counts of 10 aseptically drawn milks.

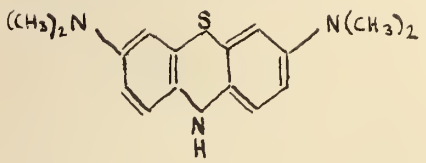
Sample	Methylene blue reduction time.	Leucocytes per cc.	Bacteria per cc. microscopic count.	Bacteria per cc. plate count.
1.	15-00.	1,740,000	64,000	1,000
2.	15-00	840,000	91,000	150
3.	12-00	1,940,000	140,000	--
4.	8-30	3,450,000	363,000	8,350
5.	7-15	3,500,000	48,000	1,350
6.	6-45	180,000	6,000	--
7.	6-15	860,000	--	50
8.	5-00	2,100,000	154,200	7,600
9.	5-00	680,000	--	100
10.	2-30	1,940,000	720,000	--

Note: Reduction times are reported in hours and minutes. Thus 7-15 means 7 hours and 15 minutes.

Thornton et al (1934) conclude, "No proof was forthcoming that the reduction of methylene blue in herd milk is related to the leucocyte content of milk." If, as evidence would indicate, in certain cases bacteria and leucocytes play only a minor part in the process of reduction then it is necessary to attribute a major role to the constituents of the milk. No system or systems in milk that would perform this function have as yet been established. Many theories have been advanced, but for the most part have lacked practical evidence.

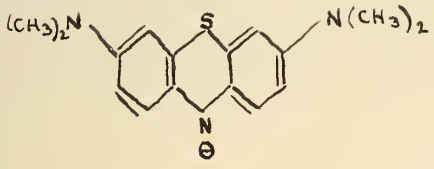
The original conception that methylene blue is reduced to the leuco form by the addition of two atoms of hydrogen must be modified to conform to the theory that oxidation-reduction processes are explicable in terms of electron migration. Michaelis' (1930) theory as to the reduction of the dye seems the most logical. He explains the process in the following steps: —

(1) Leuco Methylene Blue



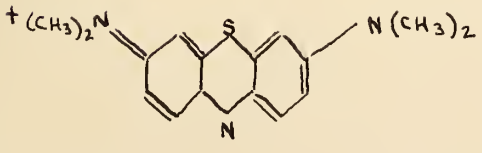
→ minus 1 H^+ – ion →

(2) Univalent anion of Leuco Methylene Blue.



minus 2 electrons

(3) Cation of Methylene Blue.



Michaelis says, "This example is of very great interest. For oxidation here cannot consist of the detachment of two H-atoms. However the action be viewed, leuco methylene blue always gives off only one H-atom and in addition one electron. Oxidation is not equivalent to loss of two H-atoms, and cannot be looked upon as a simple dehydrogenation. On the other hand, the view that oxidation in the dyestuff systems consists of loss of two electrons is indisputably valid. Wieland's view that oxidation involves only occasionally an addition of oxygen, but mainly the liberation of hydrogen, is thus seen to appear insufficient. In his scheme loss of electrons has no place, and yet this is at least for the reversible systems, the simplest and only practicable interpretation of oxidation." Thus the electronic concept provides a simple method of studying this reversible oxidation-reduction process in milk, that of the electrode potential.

TECHNIQUE

All methylene blue reduction tests, unless otherwise stated, were conducted according to the technique prescribed by Standard Methods of Milk Analysis (1929). The constant temperature water-bath used in this work was electrically heated and thermostatically controlled. The temperature in different parts of the water-bath did not vary more than half a degree. The temperatures used throughout were 37-38° C. unless otherwise stated. Direct microscopic examination of milk for bacteria and leucocytes was done according to Standard Methods of Milk Analysis (1929).

In the potentiometric work a type "K2" Leeds and Northrup potentiometer and a saturated potassium chloride-calomel half-cell were used. Burnished platinum foil was used for electrodes. All oxidation-reduction potential measurements were made at 37-38° C., unless otherwise stated, a new potassium chloride agar bridge being used in each instance.

OXIDATION-REDUCTION POTENTIALS IN MILK.

(1) Under Aerobic Conditions.

The oxidation-reduction potentials of a number of milks were followed. Fig. 1 shows a typical potential-time curve of a sample of market milk. The results are in close agreement with potentials observed by other workers.

(2) Under Anaerobic Conditions.

Introduction.

The postulation of an oxidation-reduction system or systems in milk capable of reducing methylene blue necessitates some critical examination of milk in situ before coming in contact with atmospheric oxygen. No reference could be found as to the oxidation-reduction potentials of milk in situ or of anaerobically drawn milk. It was observed that when milk is drawn into a partially exhausted tube, in which there is methylene blue, there is rapid reduction of the dye, the time taken for reduction varying from a few seconds to as many minutes. The same milk drawn aerobically would not reduce the dye, in any particular case, in less than eight hours. The milks were examined microscopically and appeared normal as far as numbers of bacteria and leucocytes can be considered as criteria. This phenomenon of dye reduction by anaerobically drawn milk is at once extremely significant, in that it indicates the presence of substances in milk which are capable under anaerobic conditions of reducing methylene blue, but which on exposure to air lose that power or only exhibit it to a much smaller degree. Thus it was considered essential to determine the oxidation-reduction potentials of milk under

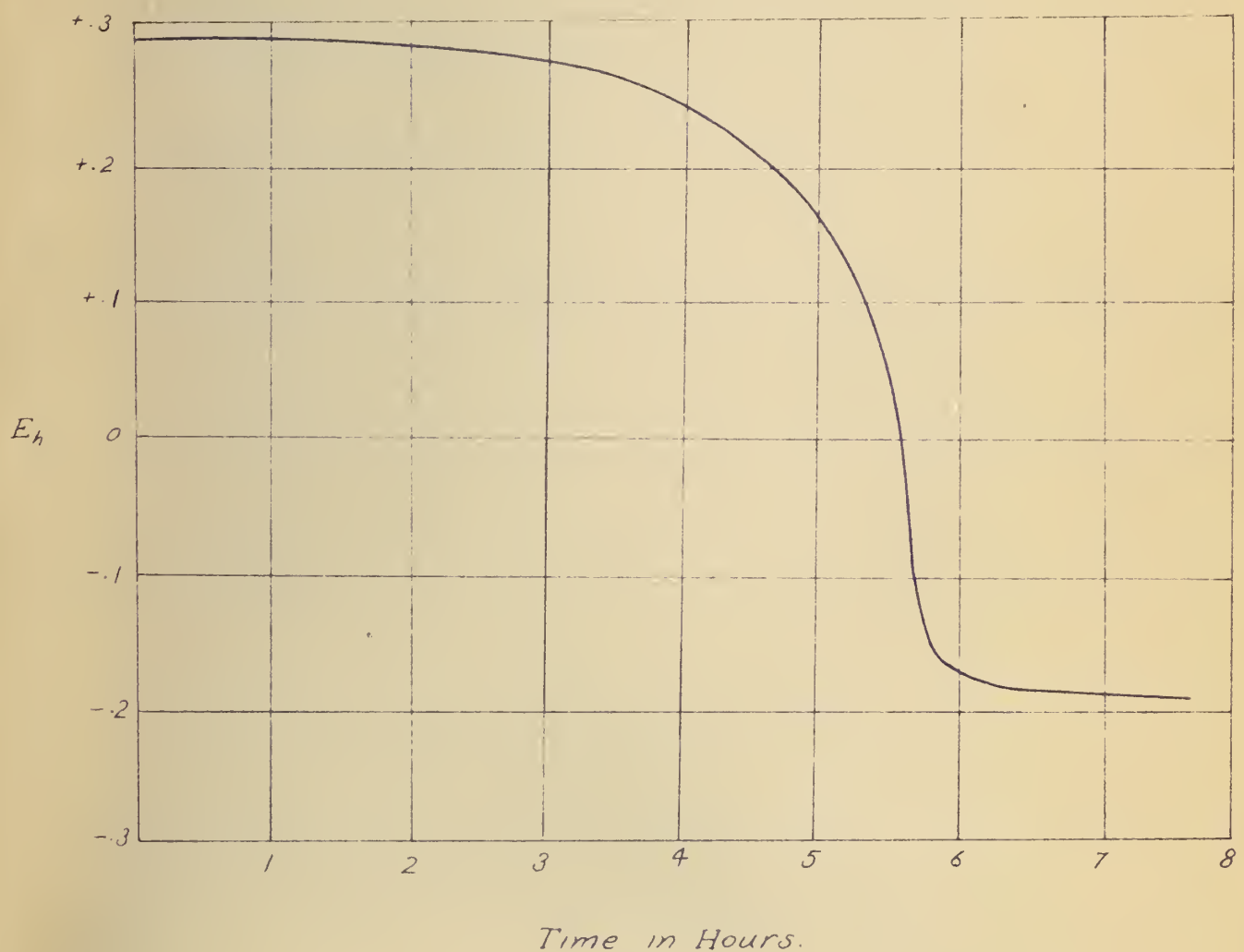


Fig. 1 - The Potential - Time Curve of a Sample of Market Milk.

anaerobic conditions.

Since 1895 attempts have been made to draw milk anaerobically. The early attempts of Hoppe, Stechnow and Pflüger are critically reviewed by Marshal (1902). Van Slyke and Baker (1919) describe a method they used in their estimation of carbonic acid and carbonates in milk but since the milk fills their containing vessel by displacement of air it must of necessity come in contact with air and therefore their technique was considered inadequate.

Technique for the anaerobic drawing of milk.

Samples were drawn under oil by hypodermic syringe in a manner similar to that used for the collection of blood but while appearing comparatively satisfactory for use with oxidation-reduction indicators, the method is open to serious criticism in that it is often impossible to exclude all air bubbles. There is also diffusion of oxygen in the oil. Accordingly a simple efficient method was devised, an adaptation of the apparatus used by Austin et al (1922) for the collection of blood. A heavy glass tube 3/4 inch in diameter and 6 inches in length (see diagram) was fitted at the bottom with a stop-cock and at the top with a three-way capillary stock-cock to which was attached about a foot of pressure tubing at the end of which was a milk tube. By manipulation of the mercury air was driven out of the system and the container-tube filled with mercury up to the three-way stop-cock. Several streams of milk were then expelled from the quarter by hand to ensure securing a normal sample: the milk tube, previously sterilized in alcohol, was then carefully inserted in the teat canal and held in place. The three-way stop-cock was so adjusted that the first milk escaped through the vent, there being sufficient force within the udder to expel it, about 20 cc. of milk was discarded in this manner,

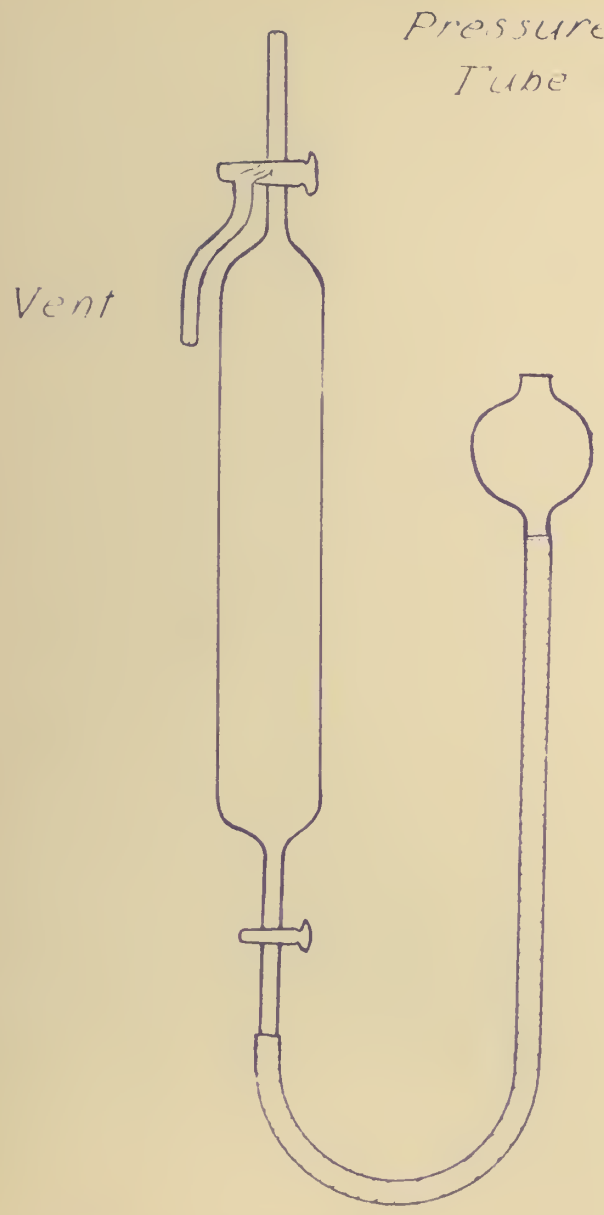


Diagram of Apparatus for the Anaerobic
Withdrawal of Milk.

sufficient to ensure that all air had been flushed out of the pressure tubing. The mercury leveller was then lowered and the stop-cock adjusted so that the milk flowed into the container as the mercury ran out. The advantages of this method are three-fold, for milk may thus be drawn into an oxidation-reduction indicator or, once drawn, may be either transferred into an electrode vessel and the potential determined electrometrically or into a Van Slyke apparatus for gas analysis.

Colorimetric determination of Oxidation-Reduction potentials under Anaerobic conditions.

Technique.

Indicator was placed in the container vessel and de-aerated as much as possible by shaking under Torecellian vacuum, milk being then drawn by the usual procedure. The concentrations of the indicators in aqueous solution were such that 1 cc. in 10 cc. of milk would give sufficient colour to ensure recognition of reduction. In this manner seven indicators were used and Table 2 records the results.

TABLE 2

The effect of anaerobically drawn milk on seven oxidation-reduction indicators.

Indicator			
M. Cresol Indophenol	E_o (pH 6.8)	+ 0.221	Reduction .30-40 secs. (slight poising.)
O. Cresol Indophenol	" "	+ 0.204	Immediate Reduction.
Thymol Indophenol	" "	+ 0.185	Immediate Reduction.
Indo 2:6 dibromophenol	" "	+ 0.135	Reduction .30-40 secs. (slight poising.)
Methylene Blue	" "	+ 0.017	Reduction .1-30 mins. (poising)
Potassium indigo tetrasulphonate	" "	- 0.037	Reduction.1-45 mins.
Potassium indigo trisulphonate	" "	- 0.072	No reduction in 2 hours.

It will be seen from the aforegoing table that four of the indicators used, even in dilute concentration, over-poised the system and that the time taken for the milk to come into equilibrium with the indicator varied from thirty seconds to forty five minutes. Thus it would appear that milk under anaerobic conditions while having a fairly well defined level of potential is not well poised and has only a small oxidation-reduction capacity.

Electrometric determination of oxidation-reduction potentials under anaerobic conditions.

Technique.

Attempts to draw milk from the udder directly into an evacuated electrode vessel were unsuccessful with one exception, as the agar bridge collapsed under reduced pressure. In the once case, a sample of abnormal milk from a cow suffering from mastitis was taken and an initial potential of -0.0395 was observed which on exposure to air became +0.2013. For subsequent determinations an all-glass electrode vessel was constructed (see diagram) by Dr. E. Boomer to whom the author is deeply indebted. It consisted simply of a tube with a stop-cock at either end.

In the tube was fused a platinum electrode and a glass tube to hold potassium chloride agar. Milk was drawn anaerobically in the manner previously described. The milk tube was then removed and the pressure tubing attached to stop-cock A. of the electrode vessel (see diagram). Nitrogen, which had been passed over hot copper, was then introduced through the side arm of the three way stop-cock and allowed to flush the electrode vessel, the three way stop-cock was then turned and the milk forced up into the electrode vessel under mercury.

A number of determinations were made in this way but the potentials obtained were not as low as would be expected from the color metric determinations. It is probable that the technique for transferring the sample

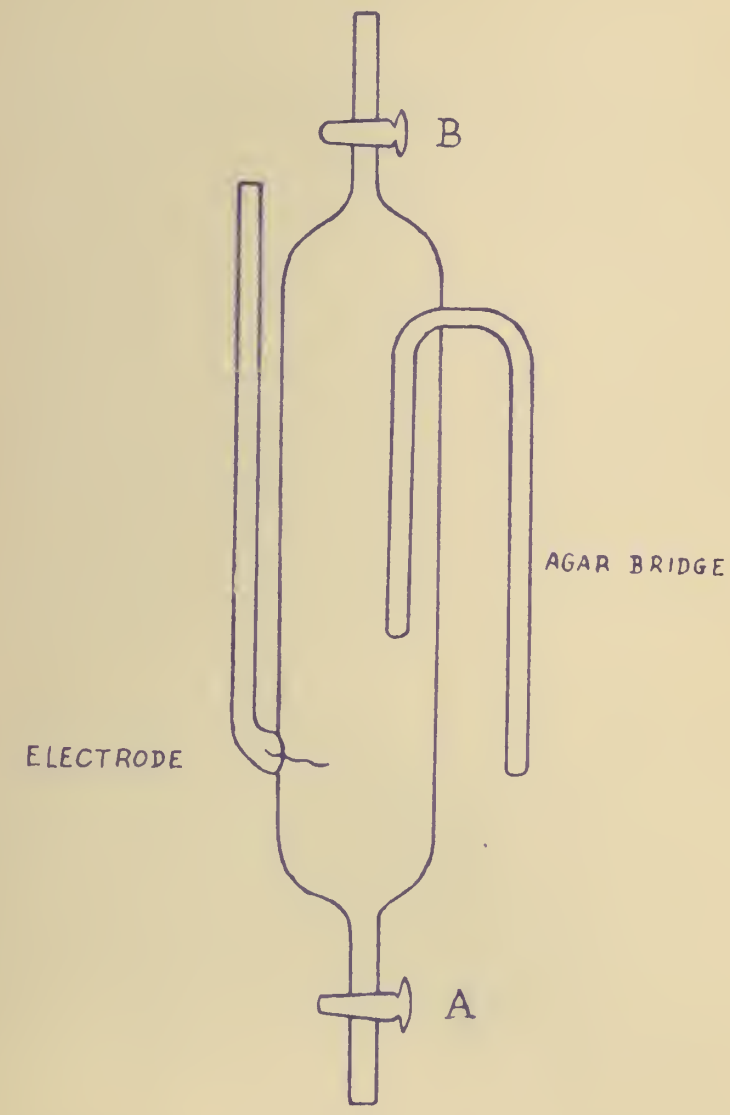


DIAGRAM OF ELECTRODE VESSEL.

from the container to the electrode vessel did not exclude all atmospheric oxygen. The lowest potential obtained in this manner was + 0.1201 and the highest + 0.1895. On admitting air the potentials became immediately more positive and in the range of fresh anaerobically drawn milk as shown in Table 3.

TABLE 3

The initial potential of five anaerobically drawn milks and the sugsequent potentials after admitting air.

Sample	Initial E_h	E_h after exposure to air.
1.	+ 0.1202	+ 0.2903
2.	+ 0.1563	+ 0.2805
3.	+ 0.1825	+ 0.3081
4.	+ 0.1865	+ 0.2803
5.	+ 0.1895	+ 0.2982

A brief consideration of oxidation-reduction systems and the use of oxidation-reduction indicators is desirable at this point. Methylene blue has a narrow range over which it reduces, its E_o at $_pH$ 6.8(30°C) being + 0.017. In observing the oxidation-reduction potentials of normal milk under aerobic conditions visual reduction of the methylene blue took place when the E_h at the electrode was as high as + 0.2, which confirms the findings of Thornton and Hastings (1929) who report complete visual reduction in different milks at E_h values between + 0.075 and + 0.225 and suggest that this increased range is due to salt effect or the influence

of other oxidation-reduction system or systems. They conclude, however, that "We wish to point out that, at present, caution should be used in interpreting reduction intensities in organic complexes in terms of potential on the basis of dye reduction."

It is not uncommon to observe visual reduction in portions of a tube while other parts show no sign of reduction which would suggest that there are different reduction intensities in different parts of the sample. Accordingly four electrodes were placed in the same sample of milk to which methylene blue had been added and the potentials determined. The accuracy of the electrodes was previously determined against a standard phosphate buffer and showed a variance of not more than 0.001 volts. Table 4 records the results.

TABLE 4

The oxidation-reduction potentials of a sample of market milk determined by four different electrodes.

Time in Hours.	E_h 1.	2.	3.	4.
Initial reading	+ 0.2867	0.2968	0.2969	0.2876
After 1 hour	0.2560	0.2769	0.2662	0.2567
2	0.2493	0.2672	0.2532	0.2462
4	0.2390	0.2562	0.2500	0.2388
6	0.2360	0.2455	0.2224	0.2288
8	0.222	0.2302	0.1928	0.1824
10	0.2000	0.2136	0.1129	0.1701
12	0.1019	0.0876	0.0654	0.0778
13	- 0.1983	-0.1976	-0.2001	-0.1980

Examination of the table will show that quite a wide variance in potential occurred in different parts of the milk until the negative limit was reached when there was approximate agreement. Visual reduction was complete at the end of the twelfth hour. This variance in potential in different parts of the milk is not surprising in a biological fluid in which the factors causing that potential are not evenly distributed. The uneven distribution of the bacteria throughout the milk is an excellent example of this fact.

That the reduction capacity of milk under aerobic conditions is comparatively large is illustrated by the fact that the concentration of the dye used in the methylene blue reduction test may be doubled with but a slight increase in the reduction time. This is not true of milk under anaerobic conditions where the addition of a slight excess of the dye is capable of oxidising the system without itself being appreciably reduced. It was noticed that anaerobically drawn milk would reduce dilute concentrations of methylene blue almost immediately but an increase in the concentration of the dye caused an increase in the time taken for reduction. In fact a concentration may be reached where there is no visual reduction of the dye. It is not unusual to attribute such behavior in a dye to its "poising action" - a term which is analogous to "buffer action" in hydrogen ion concentration. No explantion, however, has been advanced to indicate the mechanism of "poising action" in dyestuffs. More recently "poising action" has been considered as the capacity term in oxidation-reduction systems. Hewitt (1933) says, "It must be emphasized, however, that E_h is a measure of intensity level and not of capacity. In this E_h resembles temperature and $_pH$ and as temperature and $_pH$ give no information as to heat capacity and buffering powers respectively, so E_h is independant of

"poising effect", the capacity term in oxidation-reduction systems. This follows from the derivation of E_h which is dependant of the ratio of oxidised and reduced forms of the substance studied and not on their absolute quantities. Thus a 90 per cent oxidised system will have the same electrode potential no matter whether the total concentration is 0.01 per cent or 10 per cent, but the poising will be 1,000 times greater in the latter case. It is particularly important to bear these facts in mind when dealing with biological systems, many of which have well-defined electrode potentials but are not well poised." This theory presents a reasonable interpretation of the action of dyestuffs in oxidation-reduction systems and in the light of our present knowledge is, in the opinion of the writer, the only practicable explanation. Examination of Table 2 will show that the first indicator used, M. Cresol Indophenol, exhibited slight "poising" while the second indicator, O. Cresol Indophenol (which has a more negative E_o) reduced at once. This is explicable by the fact that the concentration of the first dye was four times that of the second.

THE ACTION OF METABOLITES

(1) Sulphydril Compounds.

Introduction.

Since the discovery of the function of glutathione in oxidation-reduction processes in animal tissue it has been assumed that sulphydril compounds are present in milk and that they play an important part in the reduction of methylene blue. It has become customary within the last few years to refer to the reducing substance in milk responsible for the reduction of methylene blue as "probably glutathione". Reported isolation of certain sulphydril compounds in milk necessitate an investigation into their occurrence and their affect upon methylene blue reduction times.

In the present work nitroprusside tests were run on a large number of normal milks, but in no case was there a positive test for the sulphydril group. If, however, after saturation with ammonium sulphate, sodium cyanide was added and the milk allowed to stand a few minutes before adding the sodium nitroprusside, a faint but positive reaction occurred in all cases. It was at first thought that the responsible substance was probably in solution in the oxidised form and that it was reduced by the sodium cyanide. In which case it should be possible to precipitate the sulphydril group according to standard procedure. Protein free filtrates of milk were prepared by precipitating the casein with N. acetic acid and the lactalbumin and lactoglobulin with heat, the resulting clear filtrate giving a negative Biuret. Sufficient ammonium hydroxide was added to precipitate the phosphates and the supernatant brought back to the acid side and precipitated with a slight excess of lead acetate. The lead precipitate

was decomposed by titurating with .2 N. sulphuric acid. Each step was followed by the nitroprusside test, with and without the addition of sodium cyanide, while positive in the original sample treated with sodium cyanide the nitroprusside was negative in the protein free filtrate and in each subsequent step. The active substance would then appear to be either in or adsorbed to the protein. To determine the action of sulphydril compounds in free solution in milk 1 mg. each of reduced glutathione and cysteine hydrochloride in aqueous solution was added respectively to two 20 cc. samples of milk. Both gave a nitroprusside of greater colour intensity than that of milk treated with sodium cyanide. The samples were then subjected to the procedure outlined above. Both substances remained in solution in the protein free filtrates, were not adsorbed to the protein, were quantitatively precipitated by lead acetate and were freed by decomposition of the lead precipitate with 2 N. sulphuric acid. It is well known that egg albumin, which normally gives no nitroprusside test, will do so when heated and thus denatured. If sodium cyanide is added to egg albumin the latter is denatured and gives a nitroprusside test. In a similar fashion an aqueous suspension of sodium caseinate will not give a nitroprusside test but on the addition of sodium cyanide and subsequent denaturation will do so. It would appear then that the sulphydril compound which gives the nitroprusside test in milk treated with sodium cyanide is not in free solution, but is in all probability the cystine in the protein complex, the sodium cyanide denaturing the protein and causing a cleavage of the S-S linkage leaving an -SH group exposed. Of the three principal proteins now known in milk, lactalbumin is the richest source of cystine it being present to the extent of 1.73 per cent, while in casein it is present only in 0.25 per cent. Martini (1931) states that there is

twice as much glutathione in goat's milk as there is in cow's milk. It is interesting to note that the lactalbumin content of cow's milk is 0.52% while that of goat's milk is 0.99%, or nearly twice as much, while that of human milk is 1.23%.

Five cc. samples of the three different milks (cow's, goat's and human) were saturated with ammonium sulphate and denatured with 2 cc. of sodium cyanide and the intensity of the coloration observed on the addition of sodium nitroprusside. The reaction with Folin's uric acid reagent was also observed. The comparative intensity in reaction of the three different milks with the reagants mentioned and their protein content is tabulated below:

TABLE 5.

Some particular comparisons of cow's, goat's and human milk.

	Cow	Goat	Human
Protein.			
Casein	2.95	3.04	0.91
Lactalbumin	0.52	0.99	1.23
Nitroprusside (sodium cyanide technique)	+	+++	++++
Folin's uric acid reagent	+	+	++++

The human milk was eleven days post partum and visibly free from colostrum; the goat's milk was from the mid-lactation period and the cow's milk a mixed vat sample from a city milk plant.

The effect of the addition of certain sulphydril compounds on the methylene blue reduction times and the oxidation-reduction potentials in milk was observed. Cystine, Cysteine hydrochloride, reduced Glutathione and Ergothioneine were used.

Cystine.

Since cystine is only slightly soluble in a broad zone on either side of neutrality, it was added to milk by weight. It had no effect on either the methylene blue reduction times or the oxidation-reduction potentials of milk.

Cysteine Hydrochloride.

Cysteine hydrochloride was added to milk in varying quantities. Table 6 shows the effect on the methylene blue reduction times of the addition of 5, 2 and 1 mgs. of cysteine hydrochloride in aqueous solution.

TABLE 6

The methylene blue reduction times of duplicate samples of market milk with and without the addition of cysteine.

Sample	Reduction time.
Control A.	9-40
	9-30
Control B.	8-30
	8-30
A + 5 mgs. of Cysteine	0-45
	0-45
B + 5 mgs. of Cysteine	0-40
	0-50
A + 2 mgs. of Cysteine	1-45
	1-45
B + 2 mgs. of Cysteine	1-30
	1-35
A + 1 mg. of Cysteine	2-30
	2-30
B + 1 mg. of Cysteine	2-15
	2-15

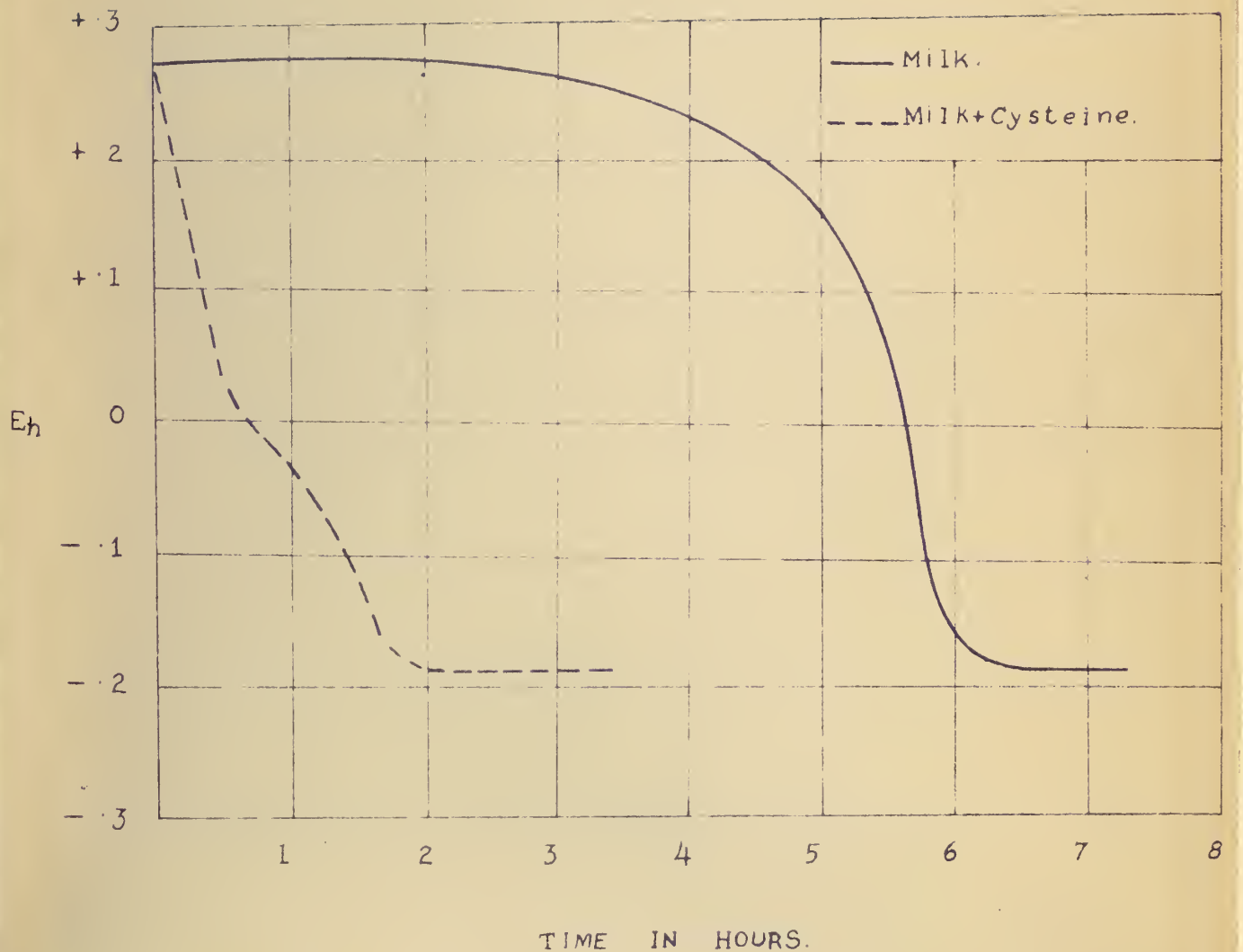


Fig 2 Potential-Time Curves of a Market Milk With and Without the Addition of Cysteine

Figure 2 illustrates the potential-time curve of a sample of market milk with and without the addition of 5 mgs. of cysteine hydrochloride in aqueous solution. It will be seen that the potential starts to drop immediately but that its negative limit coincides with that of the control.

Glutathione.

Reduced glutathione was added to milk in varying quantities. Table 7 shows the effect on the methylene blue reduction times of the addition of 5, 2 and 1 mgs. of reduced glutathione in aqueous solution.

TABLE 7

The methylene blue reduction times of duplicate samples of market with and without the addition of glutathione.

Sample	Reduction time.
Control A.	7-30 7-30
Control B.	8-15 8-20
A. + 5 mgs. of glutathione	1-00 0-55
B. + 5 mgs. of glutathione	1-00 1-00
A. + 2 mgs. of glutathione	2-50 2-55
B. + 2 mgs. of glutathione	3-00 2-50
A. + 1 mg. of glutathione	4-10 4-00
B. + 1 mg. of glutathione	4-25 4-25

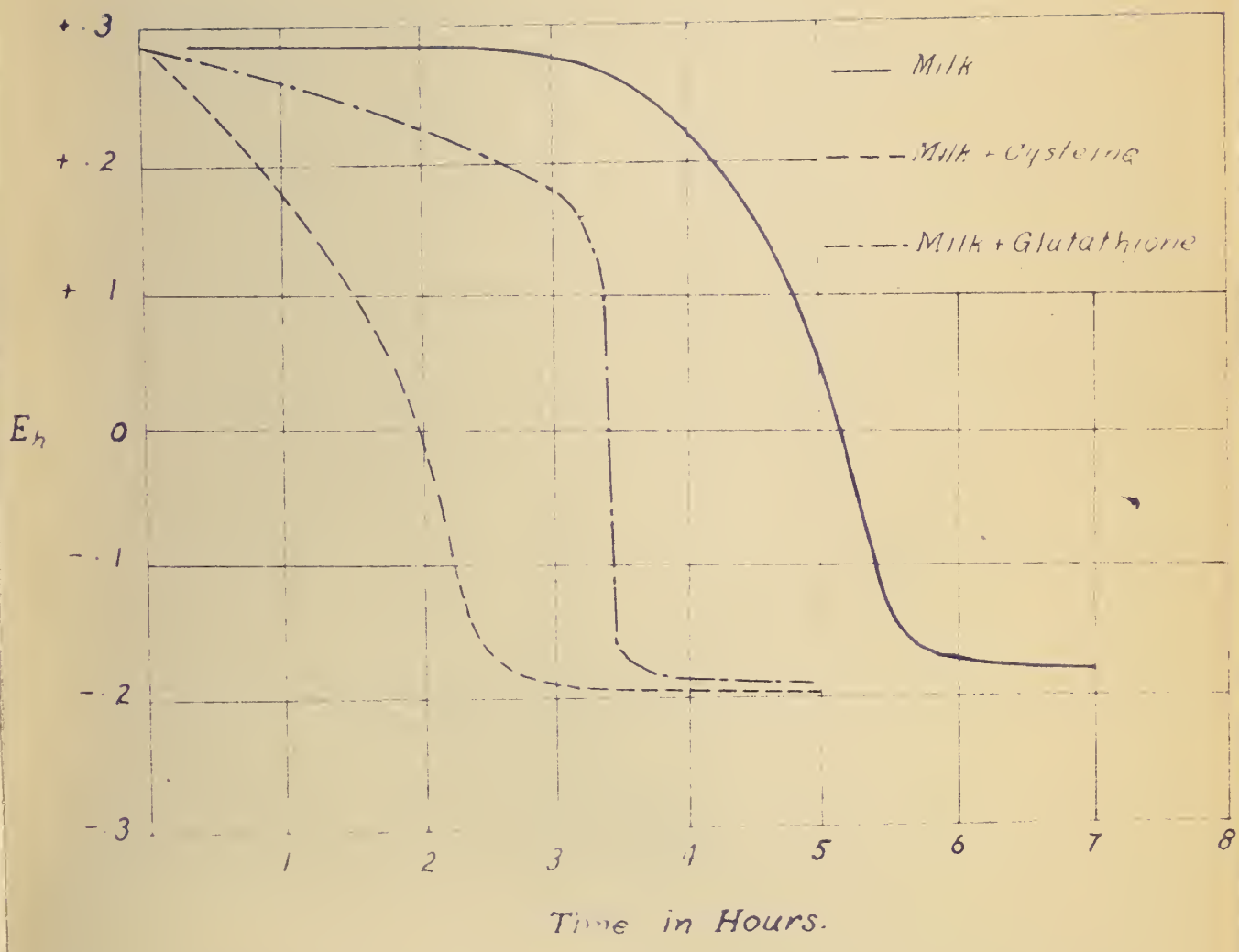


Fig. 3.- Potential-Time Curves of a Market Milk With and Without the Addition of Cysteine and Glutathione.

Figure 3 illustrates the potential-time curve of a sample of market milk with and without the addition of 2 mgs. of cysteine hydrochloride and 2 mgs. of reduced glutathione respectively. It should be noted that the glutathione will only have approximately half as many -SH groups as are present in the cysteine, thus the difference in time between the drop in potential of the two samples is explicable.

Ergothioneine.

Hunter's (1928) diazo-test for ergothioneine was run on milk with negative results. The addition of ergothioneine to milk caused no change in the methylene blue reduction times or in the oxidation-reduction potentials.

(2) Lacto-flavin.

Introduction.

The presence of a water soluble pigment in milk has been recognized for a great number of years. It is responsible for the greenish yellow colour of whey and was originally termed lactochrome. Now termed lacto-flavin it has recently been prepared from whey in crystalline form and has undergone considerable investigation by Kuhn and Wagner-Jauregg (1933) and Ellinger and Koschara (1933). Its importance from a nutritional point of view is now under investigation by a number of workers as the pigment is apparently part of the vitamin B. complex. Sufficient work has been done to show that lacto-flavin is a highly reversible oxidation-reduction system and that it is photo-chemically active. It was thought advisable to make some estimate of the effect of lacto-flavin on the

reduction of methylene blue in milk.

Preparation of an aqueous solution containing lacto-flavin from whey.

A method based on the procedure of the workers mentioned above was followed. Ten litres of whey were shaken for two hours with 240 gms of kaolin. The kaolin was taken off in about a litre of the whey and centrifuged clear, the centrifugate was washed with water until the washings were free from lactose and then eluted by shaking for one hour with a mixture of 270 cc. of 85% methyl alcohol, 270 cc. of pyridine and 1080 cc. of water and allowing to stand over night in an ice box. It was then centrifuged and the supernatant concentrated in vacuo to about 200 cc., an equal volume of 85% methyl alcohol was added to precipitate the colloidal kaolin, this process being repeated twice. The final aqueous extract was washed twice with ether to remove carotinoid pigments and a litre of acetone added causing an immediate precipitation of lactose. This was then concentrated in vacuo to about 50 cc. and was again treated with acetone, the final concentration in vacuo was an aqueous solution of about 10 cc. During the entire preparation care was taken to exclude as much light as possible. The final solution ($_{p}H$ 5.0) was a clear yellow colour with a green fluorescence; it contained lactose and gave a positive test with Folin's uric acid reagent. The effect of the addition of this extract on the methylene blue reduction times of milk was observed.

TABLE 8

The methylene blue reduction times of samples of market milk with and without the addition of varying quantities of an aqueous solution containing lacto-flavin.

Sample	Reduction time.
Control	N.R.7-00
Control + 1 drop of extract	0-30
Control + 2 drops of extract.	0-30
Control + 3 drops of extract.	0-25
Control + 4 drops of extract.	0-25.

Since the behaviour of lacto-flavin is apparently controlled by $_pH$ the aqueous solution was added to samples of milk whose $_pH$ was roughly adjusted between $_pH$ 6.0-8.6.

TABLE 9

The methylene blue reduction times of samples of market milk at different $_pH$ with the addition of an aqueous solution containing lacto-flavin.

Sample		Reduction time.
Control	$_pH$ 6.8	N. R. 7-00
" + 1 drop extract	6.0	N. R. 7-00
" " "	6.5	N. R. 7-00
" " "	6.9	N. R. 7-00
" " "	7.3	4-00
" " "	7.5	4-00
" " "	8.0	3-00
" " "	8.2	3-00
" " "	8.4	4-00
" " "	8.6	4-00

N. R. indicates no reduction.

Four days after preparation the extract became inactivated and had no effect on methylene blue reduction times either when added in large quantities or when the $_pH$ of the milk was adjusted.

A second extract was prepared from a large quantity of whey (175 litres) and was a week in preparation. The effect of the addition of this extract on the methylene blue reduction times of milk was observed.

TABLE 10

The methylene blue reduction times of samples of a market milk with and without the addition of varying quantities of an aqueous solution containing lacto - flavin.

Sample	Reduction time.
Control	N. R. 7-00
Control + 1 drop of extract	2-30
Control + 2 drops of extract	2-00
Control + 3 drops of extract	1-30
Control + 4 drops of extract	1-30

On the second day after preparation this extract lost its activity and would not cause reduction of methylene blue in milk, in fact the addition of one drop of the extract inhibited reduction for, whereas two duplicate controls reduced in ten hours, tubes to which one drop of the extract had been added did not reduce or show any signs of reduction in that time. Attempts to restore the activity of the extract by reducing it in alkaline solution with hydrogen sulphide or sulphur dioxide were unsuccessful.

The fact that an extract can be prepared from whey which catalyses the reduction of methylene blue in milk warrants further investigation along this line. New and simpler methods for the preparation of lacto-flavin from whey powder has been developed by Itter et al (1935) which should be more satisfactory for investigation along this line.

Note: The contents of Table 9 are not entirely satisfactory as no control tubes of milk with their $_pH$ adjusted and without the addition of the extract were run.

(3) Lactose.

Until recently lactose was considered to be the only saccharide that occurred normally in milk, Whitnah (1931) has shown that glucose may be present to the extent of 0.35%. Lactose is a disaccharide yielding on hydrolysis d-glucose and d-galactose, its one aldehyde group belonging to the glucose end of the molecule. The presence of this aldehyde group has designated lactose as a "reducing sugar", and as such it has been considered as a possible factor in the reduction of methylene blue in milk.

The addition of lactose to milk in varying quantities had no effect on methylene blue reduction times, nor would aqueous lactose solutions reduce methylene blue under the conditions of the test. If the $_pH$ of an aqueous solution of lactose was adjusted to over 10.0 on incubation at 37-38° C. methylene blue was reduced. Since the $_pH$ of milk is fairly constant, the extreme ranges under normal conditions being between 6.4 - 7.2, it would seem improbable that lactose plays a direct part in the reduction of methylene blue in milk.

NOTE ON THE EFFECT OF LIGHT

The influence of light on the oxidation-reduction of methylene blue has been known for a long time. Lasareff (1912) working with methylene blue solutions in solidified gelatin found that the reduction process was reversible and that, under the influence of free oxygen the methylene blue leuco base was oxidised in the dark to the dyestuff again. Whitehead (1930) observed the catalytic effect of light on the reduction of methylene blue in milk, this has been confirmed by Aikins and Fay (1932) and Frayer (1934). Whitehead suggests that sunlight catalyses an oxidation-reduction reaction in which unsaturated fats are oxidised and methylene blue is reduced. He postulates this theory on the following data - that milk from which the fat has been removed by centrifugal separation looses its power to reduce methylene blue rapidly in the presence of sunlight, but that the addition of sodium oleate restores this activity and that sodium palmitate does not. The assumption that all the fat and only the fat is removed by the centrifuging process is not warranted. It is highly probable that centrifuging a colloidal fluid such as milk will remove other substances in addition to the fat. Aikins and Fay (1932) have followed the potential of skim milk exposed to sunlight and have found a rapid drop in potential and reduction of methylene blue. A fact which has been confirmed by the writer. They also found that sodium stearate added to skim milk exposed to sunlight caused as rapid a fall in potential as does the addition of sodium oleate. The unsaturated fats then cannot be specific for this reaction. Milk contains a number of substances which are photochemically active and for a very thorough review of the chemical action of light reference should be made to Dhar (1931).

The effect on the methylene blue reduction times of the addition of cysteine glutathione and lactose to milk exposed to varying

conditions of light was observed. The addition of lactose had no effect on the reduction times. Table 11 records the effect of the addition of cysteine.

TABLE 11

The methylene blue reduction times of duplicate samples of two market milks with and without the addition of cysteine. (1) Exposed to sunlight. (2) Placed in the dark. (3) In a constant temperature water-bath.

Sample	(1) Exposed to sunlight. Temp. 26 6°C.	(2) Placed in the dark. Temp. 23 5°C.	(3) In water-bath. Temp. 37 5°C.
Control A.	0-35	N. R.	10-00
	0-35	N. R.	10-20
Control B.	0-45	N. R.	9-45
	0-45	N. R.	10-00
A. + 5 mgs of cysteine	0-05	2-30	0-45
	0-05	2-25	0-50
B. + 5 mgs. of cysteine	0-05	1-50	0-50
	0-06	2-00	0-55
A. + 1 mg of cysteine	0-08	N. R.	2-35
	0-08	N. R.	2-40
B. + 1 mg of cysteine	0-10	N. R.	2-00
	0-10	N. R.	2-10

*N.R. indicates no reduction in 24 hours.

It will be seen from the above table that in the samples placed in the dark only the ones to which 5 mgs of cysteine were added reduced, the addition of 1 mg was not sufficient to cause a drop in potential through the range of methylene blue. The exceedingly strong catalytic effect of the sunlight in the presence of cysteine is evident. The experiment was repeated using reduced glutathione and similar results were obtained.

No attempt has as yet been made to determine the wave length

of the light responsible for this reaction. Whitehead has suggested that the active agent is ultra-violet light. This seems highly improbable as the catalytic effect of the light is not impaired by passing through several layers of ordinary glass. An experiment was conducted to justify this conclusion, samples of milk with methylene blue in ordinary glass test tubes were exposed to a quartz mercury-vapour lamp for two hours, all other light was excluded: none of the samples was reduced or showed incipient reduction. The writer at the present is investigating the problem of light absorption using light filters, a communication in this regard will be made at a later date.

The question of the catalytic effect of the sunlight on the reduction of methylene blue in milk needs more investigation both from the point of view of the light absorbed in the process and the substances in the milk which are photo-chemically activated. Until a clearer knowledge of this phenomenon of reduction is obtained the methylene blue reduction test should not be conducted in direct sunlight thus a factor, the significance of which is at the present unknown, may be excluded.

NOTE ON ABNORMAL MILKS.

Milk from the very early and the last stages of lactation, as well as milk from udders suffering from pathological conditions, is abnormal and undesirable for inclusion in city milk supplies. It is fortunate from the public health point of view that these milks as a general rule cause the rapid reduction of methylene blue and so, to a large extent, can be eliminated. The factors causing reduction in these abnormal milks are unknown but the general characteristics of such milks justify a short discussion.

The influence on composition of successive phases of the lactation period is well known;the following table, according to Leach (1907) illustrates the transition from colostrum to normal milk.

TABLE 12

Transition from colostrum to normal milk.

Time after calving.	Casein %	Albmin. %	Fat %	Lactose %	Ash %	Total Solids. %
Immediately	2.65	16.56	3.54	3.54	1.18	26.93
10 hours	4.28	9.32	4.66	1.42	1.55	21.23
24 hours	4.50	6.25	4.75	2.85	1.02	19.37
48 hours	3.25	2.31	4.21	3.46	0.96	14.19
72 hours	3.33	1.03	4.08	4.10	0.82	13.56

The albumin column really represents both albumin and globulin, the latter being present in by far the largest quantity. During the final stages of lactation the decrease in daily quantity of milk is accompanied by an increase in protein and fat percentages. Both the early and the late stages of lactation are characterized by the presence of large numbers of

leucocytes. The most commonly occurring pathological condition of the udder in the Edmonton milk shed is mastitis. Milk from udders in the acute stage of the disease may usually be recognized by excessively high leucocyte and in some instances, long-chain streptococcus counts: milk from udders thus infected are often abnormal in appearance, being thick, lumpy and often pigmented. Rosell (1933) gives a very thorough description of this condition and records the chemical changes which take place in the milk.

No attempt has been made to explain the rapid reduction of methylene blue by these abnormal milks. It was noticed, however, that a sample of milk from a cow suffering from mastitis which caused reduction of methylene blue in a few minutes gave a nitroprusside after incubation at 37° C. for eight hours, the presence of the sulphydril was probably due to the degradation of the protein by bacteria which were present in very large numbers.

The factors causing reduction of methylene blue in these abnormal milks need investigation, for it is possible that some of these factors also cause reduction in normal milk but to a much smaller degree.

CONCLUSIONS.

Barthel's theory that the reduction of methylene blue in milk takes place in two stages appears essentially sound although it has not yet been shown which constituents of milk are responsible for reduction. Milk in the udder differs from milk that has been exposed to atmospheric conditions. The rapid reduction of methylene blue by anaerobically drawn milk demonstrates the presence (inherent in milk) of a system or systems with a well defined level of potential which is negative to the potential of aerobically drawn milk. That these systems are present in small quantities is shown by their small oxidation-reduction capacity. On exposure to air they are oxidised and become positive to the reduction range of methylene blue on the E_h scale. It is probable that as the dissolved oxygen in the milk is removed either by bacterial action or by other processes these systems are reduced and become negative to the reduction range of methylene blue on the E_h scale resulting in reduction of the dye.

Sulphydril compounds are not present in milk in free solution but can be demonstrated by denaturation of the milk proteins. Such substances, if present in free solution, should be in the reduced form when the milk reaches its most negative potential (the E_h of a 0.0001 N. solution of cysteine at $_pH$ 7.4 and 38° C. is -0.21). Milk with a negative potential, however, does not give a nitroprusside test - nor does milk drawn anaerobically into a partially exhausted tube. It would appear that sulphydril compounds in free solution, although they present an attractive explanation, are not concerned with the process of reduction.

Further investigation into the effect of lacto-flavin on the reduction of methylene blue in milk is essential. Such a highly rever-

sible oxidation-reduction system that is photochemically active must be regarded as a possible factor in the reduction process.

Lactose is not considered to be a direct factor in the reduction of methylene blue in milk.

The catalytic effect of light in the reduction process is recognized but whether lacto-flavin takes part in the reaction has not been established.

It is apparent that the reduction process is not a simple one. Identification of the system or systems in anaerobic milk which cause reduction of methylene blue will lay the foundation for a clearer conception of the reduction process.

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